Developmental Biology 386 (2014) 227–236

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Induction of WNT inhibitory factor 1 expression by Müllerian inhibiting substance/antiMullerian hormone in the Müllerian duct mesenchyme is linked to Müllerian duct regression

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ARTICLE INFO

Article history:
Received 18 June 2013
Received in revised form
5 December 2013
Accepted 11 December 2013
Available online 19 December 2013

Keywords:
AntiMüllerian hormone
WNT signaling
Reproductive tract development

ABSTRACT

A key event during mammalian sexual development is regression of the Müllerian ducts (MDs) in the bipotential urogenital ridges (UGRs) of fetal males, which is caused by the expression of Müllerian inhibiting substance (MIS) in the Sertoli cells of the differentiating testes. The paracrine signaling mechanisms involved in MD regression are not completely understood, particularly since the receptor for MIS, MISR2, is expressed in the mesenchyme surrounding the MD, but regression occurs in both the epithelium and mesenchyme. Microarray analysis comparing MIS signaling competent and *Misr2* knockout embryonic UGRs was performed to identify secreted factors that might be important for MIS-mediated regression of the MD. A seven-fold increase in the expression of *Wif1*, an inhibitor of WNT/β-catenin signaling, was observed in the *Misr2*-expressing UGRs. Whole mount in situ hybridization of *Wif1* revealed a spatial and temporal pattern of expression consistent with *Misr2* during the window of MD regression in the mesenchyme surrounding the MD epithelium that was absent in both female UGRs and UGRs knocked out for *Misr2*. Knockdown of *Wif1* expression in male UGRs by *Wif1*-specific siRNAs beginning on embryonic day 13.5 resulted in MD retention in an organ culture assay, and exposure of female UGRs to added recombinant human MIS induced *Wif1* expression in the MD mesenchyme. Knockdown of *Wif1* led to increased expression of β-catenin and its downstream targets TCF1/LEF1 in the MD mesenchyme and to decreased apoptosis, resulting in partial to complete retention of the MD. These results strongly suggest that WIF1 secretion by the MD mesenchyme plays a role in MD regression in fetal males.

Introduction

Sexual differentiation of the bipotential embryonic urogenital ridge (UGR) into the testes and internal male reproductive tract is regulated by the Sex Determining Region Y (SRY) transcription factor expressed in XY gonads, which leads to the production of three hormones. The fetal testes produce and secrete both Müllerian inhibiting substance (MIS; also known as anti-Müllerian hormone, AMH), which causes Müllerian ducts (MDs) to regress, and testosterone, which promotes the differentiation of the Wolffian ducts into the internal male reproductive tract tissues, i.e., the epididymides, vasa deferentia, and seminal vesicles. In the absence of MIS, as is the case with females, MDs do not regress but continue to develop and differentiate into the oviducts, uterus, and upper vagina (reviewed in (Teixeira et al., 2001)). Defects in either the gene for MIS or its receptors can result in a form of male pseudohermaphroditism characterized by retained MD-derived tissues (Behringer et al., 1994; Mishina et al., 1996; Jamin et al., 2002). In humans, approximately 85% of patients with persistent Müllerian duct syndrome (PMDS) are thought to have underlying mutations in MIS or its receptors (Belville et al., 2004; Josso et al., 2005). The third hormone, insulin-like 3 (INSL3), is produced by the pre-and postnatal testes and is necessary for testicular descent (Nef and Parada, 1999; Zimmermann et al., 1999).

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http://dx.doi.org/10.1016/j.ydbio.2013.12.015

As with other members of the transforming growth factor β (TGFβ) super family, MIS binds to its specific type II receptor (MISR2), which activates its latent kinase, causing recruitment and phosphorylation of a type I receptor, either ACVR1 (ALK2) or BMPR1A (ALK3), to initiate a downstream signaling cascade that results in apoptosis and regression of the MDs (Teixeira et al., 2001; Josso and Clemente, 2003). The MDs first form from coelomic epithelial cells that invaginate, proliferate, and migrate in a cranial-to-caudal direction to merge with the urogenital sinus epithelium (Guioli et al., 2007; Orvis and Behringer, 2007; Fujino et al., 2009). The MDs are similarly eliminated in a cranial-to-caudal manner as a result of MIS action (Picon, 1969; Tsuji et al., 1992), which is attributable to the cranial-to-caudal expression of *Misr2* (Allard et al., 2000; Arango et al., 2008). We have recently shown that *Misr2*-expressing cells are initially present in the coelomic epithelium and subjacent mesenchyme of both male and female UGRs, but that under the influence of MIS, the *Misr2*-expressing cells proliferate and migrate into the mesenchyme surrounding the male MD (Hayashi et al., 1982; Trelstad et al., 1982; Zhan et al., 2006). We and others have also shown that expression of the MIS type I receptors, is also spatiotemporally controlled, and that the SMAD1, 5 and 8 subfamily of intracellular signal transducers are involved in MD regression (Gouedard et al., 2000; Clarke et al., 2001; Visser et al., 2001; Zhan et al., 2006; Orvis et al., 2008). with SMAD8 being predominantly expressed over SMAD1 and over SMAD5, which is poorly expressed during MD regression (Clarke et al., 2001; Zhan et al., 2006) One of the important unanswered questions in MD regression is how MISR2, which is expressed in the mesenchyme surrounding the MD but not in the MD epithelial cells at the time of regression (Baarends et al., 1994; di Clemente et al., 1994; Teixeira et al., 1996), induces apoptosis in the neighboring epithelial cells (Tsuji et al., 1992; Catlin et al., 1997; Allard et al., 2000).

WNT/β-catenin signaling is known to be crucial for normal uterine development from the MDs in females (Kobayashi and Behringer 2003). For example, *Wnt5a* knockout mice display anomalous development of the uterine horns, cervix and vagina, and the uteri from *Wnt7a* knockout mice have defective myometria, endometrial glands, and oviductal structures (Miller and Sassoon, 1998; Parr and McMahon, 1998; Mericskay et al., 2004). The MD mesenchyme-specific expression of *Wnt5A* and MD epithelium-specific expression of *Wnt7A* adds further complexity to the respective roles in uterine development (Mericskay et al., 2004). The different phenotypes observed by the knockout of either *Wnt7a* or *Wnt5a* suggests that these ligands have different functional roles, which might be attributable to the separate signaling pathways used by the respective ligands, i.e., the canonical β-catenin pathway for WNT7A (Mikels and Nusse, 2006b) and the ROR_2 receptor, the Ca^{2+} or the planar cell polarity pathways for WNT5A (van Amerongen et al., 2008). However, WNT5A can also signal through the canonical β-catenin pathway, depending on specific WNT receptor expression (He et al., 1997; Mikels and Nusse, 2006a; van Amerongen et al., 2012).

WNT signaling is not only important for uterine development in females, it is also a key factor in MD regression in male fetuses. Male *Wnt7a* knockout mice have retained MDs (Miller and Sassoon, 1998; Parr and McMahon, 1998), so far the only *Wnt* family gene knockout reported to develop this phenotype. However, *Misr2* mRNA expression is also lost in *Wnt7a* knockout mice, thereby abrogating MIS signaling and precluding any inference on its role in the downstream activity of β-catenin. Nuclear accumulation of β-catenin has been reported in the MD mesenchyme cells during MD regression (Allard et al., 2000), and male mice with conditional knockout of β-catenin from the MD mesenchyme were shown to have retained MDs (Kobayashi et al., 2011), indicating that nuclear β-catenin activity in the MD mesenchyme is necessary for MD regression. Taken together, these studies support a dual role for WNT/β-catenin signaling in MD biology, one for regression in males and another for differentiation in females. We have shown that either conditional knockout of β-catenin or constitutive activation of β-catenin in the MD mesenchyme leads to myometrial pathologies in female mice (Arango et al., 2005; Tanwar et al., 2009) and that constitutive activation of β-catenin in the MD mesenchyme predisposes male mice to focal MD retention (Tanwar et al., 2010). Thus, it appears that the contradictory finding that MD retention in males with either β-catenin knockout (Kobayashi et al., 2011) or constitutive activation of β-catenin (Tanwar et al., 2010) using the same *Misr2*-driven Cre would suggest that MD regression is exquisitely sensitive to WNT/β-catenin signaling in the MD mesenchyme or that highly localized microenvironmental factors that modulate WNT/β-catenin signaling might need to act in concert with β-catenin to complete MD regression in males. In contrast to the critical roles played by WNT/β-catenin signaling in the MD mesenchyme, its expression in the MD epithelium does not appear to be necessary for MD regression (Kobayashi et al., 2011).

We have identified WNT inhibitory factor 1 (WIF1) (Hsieh et al., 1999) as a candidate intercellular factor involved in the MIS/MISR2-mediated regulation of MD regression using microarray analyses of *Misr2* homozygous knockout UGRs compared with heterozygous controls. Here we show that MIS induces expression of *Wif1* mRNA during normal MD regression in males and that knockdown of *Wif1* by siRNA leads to partial retention of the MD epithelium in UGR assays. These results suggest that the dual roles played by β-catenin in MD retention and regression can be controlled by the local expression of WNT inhibitors such as WIF1.

Methods

Microarray analysis of Misr2 knockout and control mice

Mice used in this study were housed under standard animal housing conditions and maintained on a C57BL/6;129/SvEv mixed genetic background. The Institutional Animal Care and Use Committee at Massachusetts General Hospital approved the protocols for animal experimentations performed in this study. *YFP* mice (*Gt(ROSA)26Sor*$^{tm1(EYFP)Cos}$, obtained from Dr. Frank Costantini (Srinivas et al., 2001)) were mated with *Misr2-LacZ* mice (*Amhr2*$^{tm2(LacZ)Bhr}$, obtained from Dr. Richard Behringer (Arango et al., 2008)) to obtain homozygous *YFP/YFP;Misr2-LacZ/Misr2-LacZ* females, which were then mated with male *Misr2-Cre/+* (*Amhr2*$^{tm3(cre)Bhr}$, obtained from Dr. Richard Behringer (Jamin et al., 2002)) mice to produce *Misr2-Cre/Misr2-LacZ;YFP/+* embryos that are knocked out for *Misr2* and cannot transduce MIS signaling but express the YFP reporter or *Misr2-LacZ/+;YFP/+* embryos that can transduce MIS signaling and do not express the YFP reporter. Genotyping was performed when necessary with DNA collected from tail biopsies using standard PCR protocols. Timed pregnant matings were performed, and the presence of a vaginal plug in the morning was considered embryonic day 0.5 (E0.5) at 12 p.m. UGRs of the male embryos were sorted manually by YFP fluorescence (YFP+ were *Misr2* knockouts, YFP− were *Misr2* heterozygotes) using a fluorescence dissecting microscope, and the mesonephroi were dissected away from the gonads with a scalpel as cleanly as possible, pooled, frozen and stored at −80 °C. Female UGRs were discarded. Total RNA was prepared (~5 μg) from approximately 200 mesonephroi for each genotype, the mRNA converted to cRNA, and compared by microarray analyses using the Affymetrix oligonucleotide chips (Mouse430_2) at the Harvard/Partners Center for Personalized Genetic Medicine. Data

from the microarray analysis has been deposited in the GEO database under the accession number GSE38009.

Animals, organ culture and recombinant human MIS

UGRs were dissected from wildtype embryos of timed pregnant mice and studied at developmental stages E12.5, E13.5 and E14.5, to determine normal gene expression patterns and morphological changes in vivo. UGRs collected from timed pregnant mice at E13.5 were also dissected and then cultured on MilliCell-CM membrane (Millipore, Bellerica, MA) over 2% agarose gel droplets immersed in CMRL 1066 medium (Life Technologies, Grand Island, NY) supplemented with 10% female fetal bovine serum (Aires Scientific/Biologos, Richardson, Texas) to avoid bovine MIS in male serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 nM testosterone, which stimulates Wolffian duct development, for comparison with MDs. Cultures were carried out with or without recombinant human MIS (rhMIS) at a final concentration of 6 μg/ml (42.5 nM). MIS was purified from serum-containing media of Chinese hamster ovary clonal cells expressing native MIS by immunoaffinity chromatography as described previously (Ragin et al., 1992).

RNA probes

Digoxigenin-labeled antisense and sense riboprobes were generated by in vitro transcription using digoxigenin-labeled nucleotide mix (Roche, Indianapolis, IN) according to the manufacturer's instructions. A PCR fragment of *Wnt7a* coding sequence (NM_009527; nucleotides 335-970) was subcloned into pCRII-TOPO (Life Technologies), digested with *Eco*RV, and transcribed with Sp6 RNA polymerase to make antisense probes. A PCR fragment of coding sequence of *Wif1* (NM_011915; nucleotides 516-1131) was cloned from a mouse testis cDNA library into pCR2.1-TOPO (Life Technologies), digested with *Spe*I, and transcribed with T7 RNA polymerase to make antisense probes. The *Mis* plasmid (Swain et al., 1998) was digested with *Sma*I and transcribed with T7 RNA polymerase for the antisense probe. The probe for *Misr2* was made as previously described (Clarke et al., 2001). All templates were purified using phenol–chloroform–isoamyl alcohol and ether for RNase-free conditions.

In situ hybridization

Immediately following dissection at various gestational ages or after organ culture in RNase-free conditions, UGRs were fixed for 2 h at 4 °C in 4% paraformaldehyde (PFA). The UGRs were dehydrated and stored in 100% methanol until further use. Tissues were rehydrated, treated with hydrogen peroxide, then with 10 g/ml proteinase K, pre-hybridized, and hybridized with digoxigenin-labeled sense or antisense riboprobes overnight at 70 °C. A minimum of 3 UGRs was used for each experimental condition. After high-stringency washing, the samples were placed in 10% sheep serum/TBS-T for 1.5 h at room temperature for blocking, then incubated with anti-digoxigenin-AP antibody (Roche) at 1:2000 overnight at 4 °C with gentle rocking. The signals were visualized using the BM-Purple alkaline phosphatase substrate (Roche). For frozen sections, samples were embedded in O.C.T. compound and subsequently cryosectioned at 16 μm. Photos were taken with a Nikon SMZ1500 dissecting microscope with a Spot digital camera (Diagnostic Instruments, Sterling Heights, MI).

RNAi in organ culture

A cocktail of siRNA duplex oligoribonucleotides of *Wif1* (MSS280614, MSS280615, MSS280616) and negative control duplexes were purchased from Life Technologies. RNAi in organ culture was performed as described previously (Zhan et al., 2006). siRNA concentrations between 50 and 400 nM were tested with murine embryonal fibroblasts using the Alexa Fluor-555 siRNA control (Life Technologies) for optimal silencing efficiency and minimal toxicity; as a result, 200 nM was selected for further studies. UGRs from E13.25 were transfected with siRNA duplex in serum-free culture medium by using the Oligofectamine RNAiMAX reagent (Life Technologies). siRNAs and Oligofectamine were diluted in separate tubes in OptiMEM (Life Technologies), combined and incubated for 20 min at room temperature. The siRNA: Oligofectamine mixture was added to the medium and incubated with immersed UGRs for 12 h. The UGRs were subsequently placed on MilliCell-CM membranes to continue culture for an additional 60 h at the air-media interface over complete medium. The tissues were then fixed in 4% PFA for either whole mount in situ hybridization or immunofluorescence staining.

Immunofluorescence staining following Wif1 siRNA treatment of UGRs

For immunofluorescence staining, UGRs were incubated in 15% sucrose solution for 2 h followed by embedding in 7.5% gelatin/15% sucrose. The embedded tissues were frozen at −60 °C in isopentane and sectioned at 9 μm. For β-catenin, TCF1 and LEF1 immunostaining, serial sections were washed in PBS three times consecutively. Sections were then washed in PBST and blocked with 10% donkey serum, 1% bovine serum albumin, and 0.1% triton-X in PBS for β-catenin or 5% goat serum and 0.1% triton-X for TCF1/LEF1 in TBST, 1 h at room temperature. Tissue sections were then incubated in a humidified chamber overnight at 4 °C with anti-β-catenin primary antibody (1:250; BD Transduction Laboratories, San Jose, CA), anti-TCF1 and anti-LEF1 primary antibodies (1:500; Cell signaling Technologies, Danvers, MA). Tissue sections were subsequently washed with PBS-T, incubated for 1 h at room temperature with AlexaFluor secondary antibodies (1:250; Life Technologies), then counterstained with 4′,6′-diamidino-2-phenylindole (DAPI). Images were photographed using a microscope (Nikon Eclipse TE 2000-S) equipped with a Spot digital camera.

TUNEL assay

After transfecting E13.5 male UGRs with *Wif1* siRNA and RNAi for 12 h, they were incubated for an additional 48 h in complete medium. The UGRs were fixed in 4% PFA at 4 °C for 2 h, washed in PBS and kept at 4 °C for immediate assay or stored in 70% ethanol at 4 °C for later analysis. The UGRs were post fixed in cold ethanol: acetic acid at a 2:1 ratio for 5 min at −20 °C for permeabilization. Terminal deoxynucleotidyl transferase enzyme and anti-digoxigenin conjugates were applied according to the ApopTag Plus Fluorescein In Situ kit (Millipore) manufacturer's protocol. Counterstaining was performed with DAPI, and the UGRs were examined with a dissecting microscope. Discrete TUNEL+ cells lining the Müllerian duct were manually counted in photos of longitudinal planes obtained after identifying the section where the Müllerian duct was present along with sections approximately 100 μm above and below. As a result, 3 sections per ridge for 5 control ridges and 6 *Wif1* siRNA treated ridges were counted.

Statistical analysis

Results are expressed as mean values ± standard error of mean (SEM) from a minimum of three individual experiments. Statistical analyses were perfomed by ANOVA or unpaired *t*-test as appropriate with Prism 5 (GraphPad, La Jolla, CA). *P*-values ≤ 0.05 were considered statistically significant.

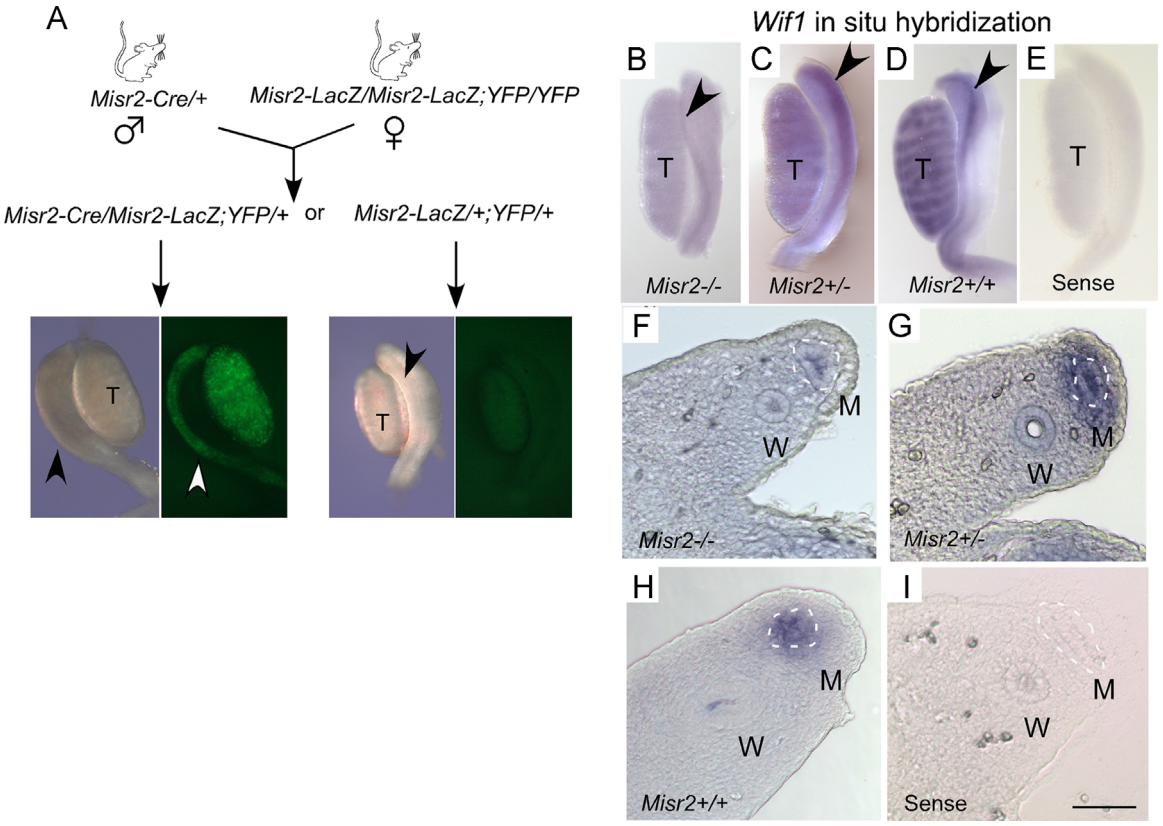

Fig. 1. *Wif1* expression is diminished with loss of MIS signaling in UGRs. (A) Schematic showing the breeding and scoring used to distinguish UGRs from *Misr2* knockout mice and heterozygous mice. *Misr2* knockout UGRs have both the *Misr2-Cre* and *Misr2-LacZ* alleles. The Cre recombinase from the *Misr2-Cre* allele drives YFP expression, which is detected by fluorescence. The *Misr2-Cre* allele is not in the heterozygotes and YFP expression is not detected. Whole mount in situ hybridization for *Wif1* mRNA in male UGRs from embryonic day 13.5 *Misr2* knockout mice (B) displays downregulation of *Wif1* as indicated by BM purple compared to *Misr2* heterozygous control (C) and wildtype (D) UGRs, where there is a clear expression along the MD as well as in the gonads. (E) No *Wif1* mRNA expression is detected with a sense RNA probe. Frozen sections of the whole mount in situ hybridized UGRs reveal noticeably higher levels of *Wif1* mRNA detection in the mesenchyme surrounding the MD epithelium of the heterozygous and the wildtype controls ((G) and (H), respectively) than in the *Misr2* knockout (F) or in the sense control (I). Arrowheads indicate MDs in whole mounts. MD epithelium is outlined with a dashed line in panels with sections. *T*=testis, *M*=Müllerian duct, *W*=Wolffian duct. Bar=100 μm.

Results

MIS induces Wif1 expression in the MD mesenchyme

The MIS ligand will not be able to bind to MD mesenchyme cells lacking the MISR2 receptor and initiate the signaling cascade required for MD regression. To determine which genes are induced by MIS signaling during MD regression, a mating scheme was developed for efficient sorting of heterozygous *Misr2* UGRs from homozygous *Misr2* knockout UGRs of E14.5 fetuses without resorting to PCR genotyping (Fig. 1A). The male gonads were dissected away from the UGRs so that only the duct-containing mesonephroi were collected and pooled for total RNA isolation enriched for agonadal mRNAs. The results of microarray analyses of the RNAs from *Misr2*-expressing UGRs compared to the *Misr2* knockout UGRs are shown in Tables 1 and 2. The loss of *Misr2* expression in the knockout RNA (not shown) was used to validate the ability of the experiment to differentiate between *Misr2* knockout and heterozygous UGRs.

Wif1 mRNA expression was induced more than 7-fold in MIS-responsive agonadal UGRs (Table 1). WIF1 belongs to the secreted Frizzled-related protein class of WNT signaling inhibitors that binds to WNTs, which in turn inhibits their binding to their receptors (Hsieh et al., 1999). Its expression and activity have been reported in a variety of different developmental pathways that, when disrupted, can lead to developmental anomalies and cancer (Kawano and Kypta 2003). Because of the importance of WNT signaling for MD differentiation and regression, we performed whole-mount in situ hybridization to verify the induction of *Wif1* mRNA by MIS. UGRs were collected from E13.5 embryos of the *Misr2* knockout and heterozygous embryos. E13.5 was chosen

Table 1
Genes upregulated with MIS signaling.

Gene name	Accession	Fold
Sp6	NM_031183.2	7.715
Wif1	NM_011915.2	7.36
Msx2	NM_013601.2	6.688
Dlx5	NM_010056.2	4.069
Gata5	NM_008093.2	3.939
Ednrb	NM_007904.4	3.633
Ablim3	NM_198649.3	3.503
Ncstn	NM_021607.3	3.451
Ddx17	NM_199080.2	3.33
Syt1	NM_009306.3	3.273
H2-T23	NM_010398.1	3.219
Mbp	NM_010777.3	3.023
Mael	NM_175296.4	2.977
Stk31	NM_029916.2	2.951
Dppa4	NM_028610.2	2.937
Rpl39l	NM_026594.2	2.914
Pou5f1	NM_013633.3	2.893
Zmat2	NM_025594.3	2.867
Nfatc1	NM_016791	2.76
Helb	NM_080446.2	2.751
Dazl	NM_010021.5	2.7
Gfra2	NM_008115.2	2.658
Epha4	NM_007936.3	2.634
Ptprd	NM_011211.3	2.576

because MD regression is well underway by E14.5, when it is more difficult to detect the MDs in males. Expression of *Wif1* mRNA was clearly visible along the entire length of the MD and in the testis cords of both *Misr2* wild type and heterozygous UGRs (Fig. 1C and D). In *Misr2* knockout UGRs, only trace expression of *Wif1* was observed (Fig. 1B) when stained for an equivalent amount of time as *Misr2*-expressing UGRs but which was still higher than that observed with a negative control sense probe (Fig. 1E). Analyses of the whole-mount UGRs in frozen sections were done to determine the spatial relationship of *Wif1* mRNA expression with respect to the MD epithelium. In the *Misr2* wild type and heterozygous UGRs, expression was localized to the mesenchyme surrounding the MD epithelium (Fig. 1G and H) where *Misr2* is expressed and to the MD epithelial cells as well. However, the sections from the *Misr2* knockout UGR showed what little *Wif1* mRNA was observed localized to the MD epithelial cells (Fig. 1F).

The expression pattern of *Wif1* mRNA over time was studied by whole mount in situ hybridization in male UGRs from E12.5, E13.5 and E14.5 embryos. At E12.5 *Wif1* mRNA expression is essentially negative (Fig. 2A) and frozen section analysis shows that the light staining in the duct could be an artifact of substrate pooling in the MD lumen (Fig. 2D). However at E13.5 male UGRs are strongly positive for *Wif1* in the mesenchyme surrounding the MD epithelium, particularly at the cranial end (Fig. 2B and E). In contrast, female UGRs at this age do not show *Wif1* expression by whole mount in situ hybridization above background (Fig. 2H and I). As MD regression progresses caudally at E14.5, *Wif1* expression was observed at the middle to caudal end of the MD where regression has not yet occurred (Fig. 2C and G). At the cranial end where MD regression has occurred, *Wif1* expression is not observed (Fig. 2C and F). In addition to MIS, the embryonic male gonads produce testosterone, which is necessary for Wolffian duct development and has been shown to induce *Wif1* expression (Keil et al., 2012), and other secreted factors that could account for induced *Wif1* expression in MD mesenchyme in male UGRs compared with females. To ensure that *Wif1* expression is induced by MIS alone, without the need for any other factor produced by the fetal male gonads, E13.5 female UGRs were exposed to recombinant human MIS (6 μg/ml) for 48 h and then analyzed by whole mount in situ

Table 2
Genes downregulated with MIS signaling.

Gene name	Accession	Fold
Fbxo10	NM_001024142.1	−2.744
Phip	NM_001081216.1	−2.764
Cnn3	NM_028044.2	−2.944
Spry4	NM_011898.2	−3.112
Hspa14	NM_015765.2	−3.21
Bcas2	NM_026602.3	−3.405
Kif1c	NM_153103.2	−4.155
Tfap2c	NM_009335.2	−4.37
Kif5b	NM_008448.3	−4.406
Mat2a	NM_145569.4	−6.663
Fank1	NM_025850.2	−8.488

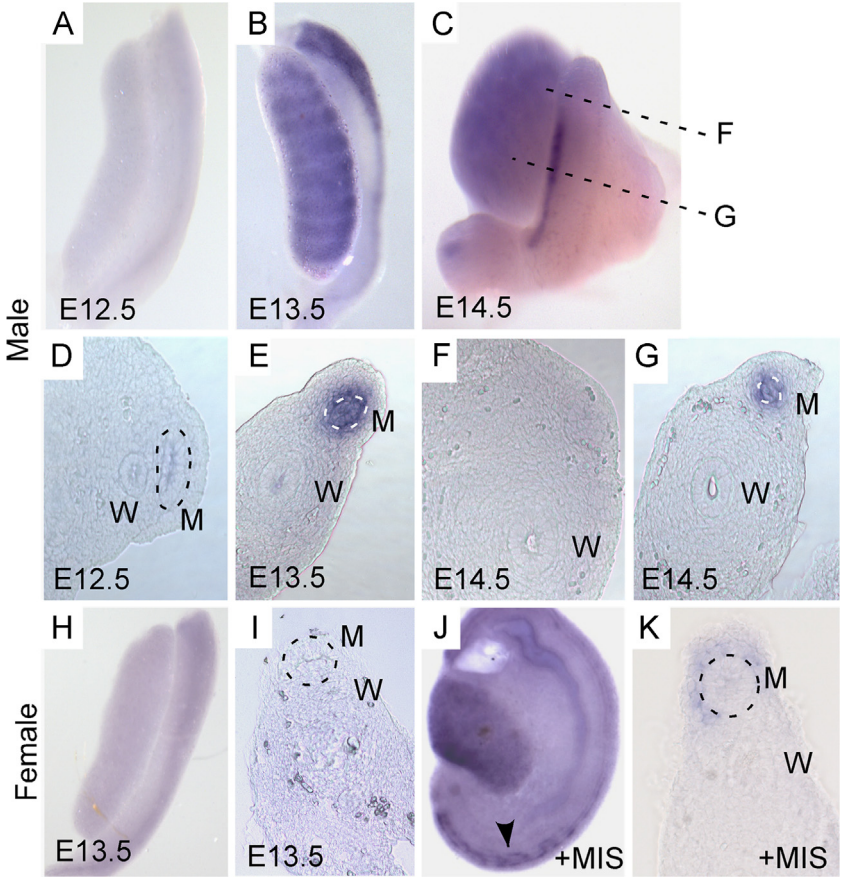

Fig. 2. MIS induces Wif1 expression. Time course analysis shows expression of *Wif1* mRNA by in situ hybridization in male UGRs at E12.5 (A), E13.5 (B) and E14.5 (C). Corresponding frozen sections ((D) and (E)) show strongest *Wif1* mRNA expression is detected in the MD mesenchyme at E13.5. At E14.5, *Wif1* mRNA is not detectable in rostral sections where the MDs have regressed ((C) and (F)) but more caudal expression is observed where the MD has not yet regressed ((C) and (G)). *Wif1* mRNA expression is low or not detectable in female UGR at E13.5 ((H) and (I)). In contrast, *Wif1* mRNA is detected in the surrounding mesenchyme of the MDs (arrowhead) in E13.5 female UGRs exposed to rhMIS for 60 h in organ culture ((J) and (K)). *M*=Müllerian duct, *W*=Wolffian duct. MD epithelium is outlined with a dashed line in panels with sections.

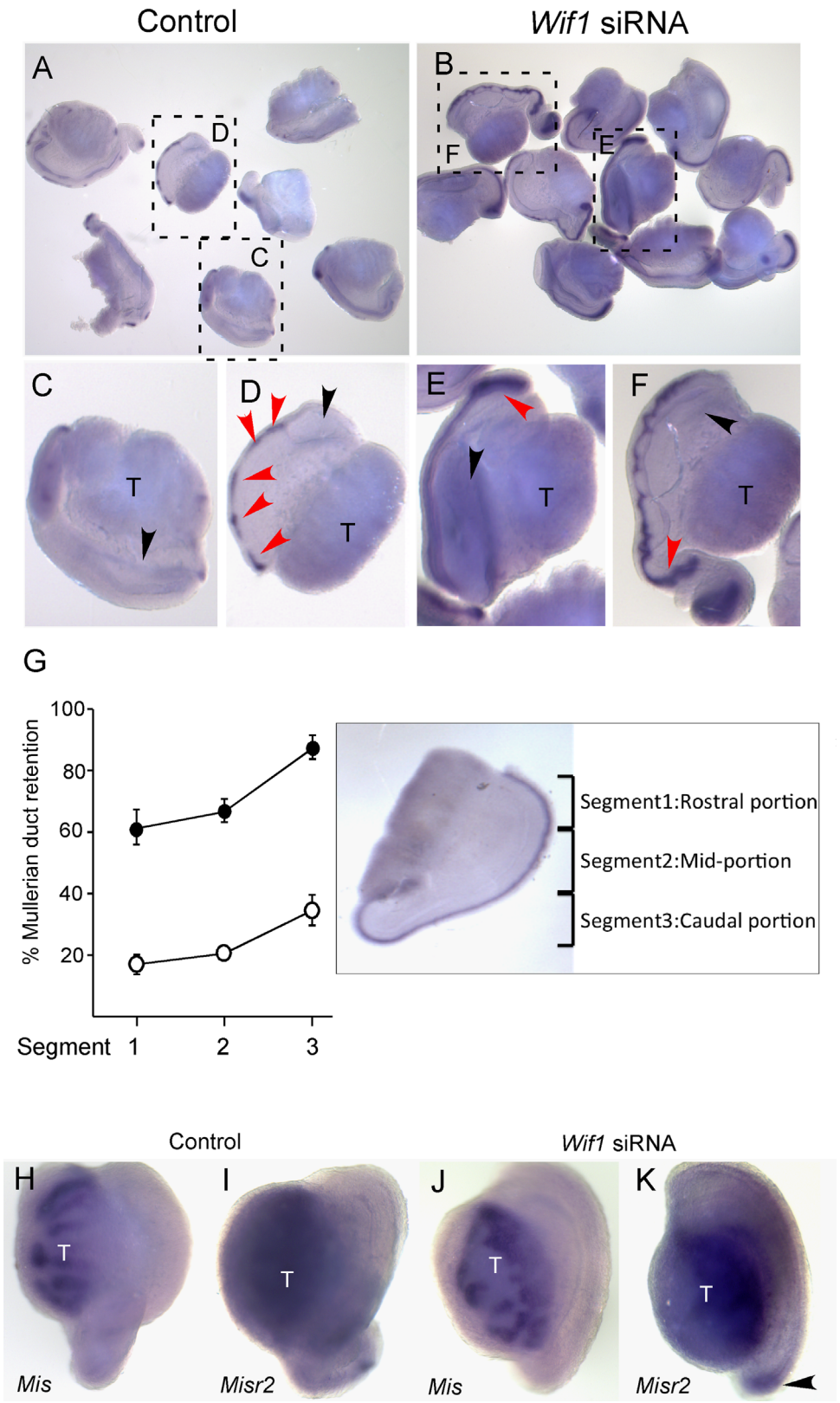

Fig. 3. *Wif1* mRNA knockdown inhibits MD regression. E13.5 male UGRs were treated with either control scrambled siRNA duplexes or with a cocktail of *Wif1* siRNA duplexes for 12 h and assayed for MD regression after 60 h by whole mount in situ for *Wnt7a*, a MD epithelium-specific mRNA. (A) The absence of Wnt7a in most of the MD epithelium indicates nearly complete regression of the MDs was observed in most UGRs with control siRNA. (B) Complete MD regression was not observed in UGRs transfected with *Wif1* siRNA. Higher magnification of representative UGRs boxed in (A) and (B) are shown in (C)–(F). Black arrowheads indicate the Wolffian duct and red arrowheads indicate *Wnt7a*-postiive MDs. UGRs from three separate sets of experiments were scored for MD retention for semi-quantitative comparison with the UGRs divided into rostral, middle and caudal segments (G). Open circles (○) are male control siRNA-treated UGRs, and closed circles (●) are *Wif1* siRNA-treated UGRs showing significantly fewer MD regression in all three segments analyzed. Neither *Mis* in the testes nor *Misr2* mRNAs in the testes and remaining MD appeared to be affected by transfection with *Wif1* siRNA ((J) and (K)) compared to controls ((H) and (I)). Arrowhead indicates remaining MD with *Misr2* mRNA. *T*=testis.

hybridization for *Wif1* mRNA. Partial MD regression was observed in female UGRs (Fig. 2J), which like male UGRs express the *Misr2* in the MD mesenchyme (Baarends et al., 1994; di Clemente et al., 1994; Teixeira et al., 1996), precluding continued expression of *Wif1* as was observed in male UGRs with MD regression (Fig. 2C). Also as with male UGRs (Fig. 2G), analysis of frozen sections where MD regression was not complete showed expression of *Wif1* in female UGRs exposed to MIS (Fig. 2K). These results clearly demonstrate the transitory nature of *Wif1* expression in a cranial to caudal manner within a narrow window of time in the MD mesenchyme during male UGR development that is induced by MIS.

Wif1 knockdown inhibits complete MD regression in vitro

To determine whether MD regression in vitro is dependent on MIS-induced expression of *Wif1*, knockdown of *Wif1* was performed with a cocktail of *Wif1*-specific siRNA in UGRs from E13.25 male embryos in organ culture. When the UGRs were treated with negative control siRNA, complete or near complete regression of the MDs was observed in the UGRs by whole mount in situ hybridization for *Wnt7a* mRNA (Fig. 3A), which is specific to the MD epithelium (Miller and Sassoon, 1998; Parr and McMahon, 1998). Occasional MD epithelium was detected, usually in the

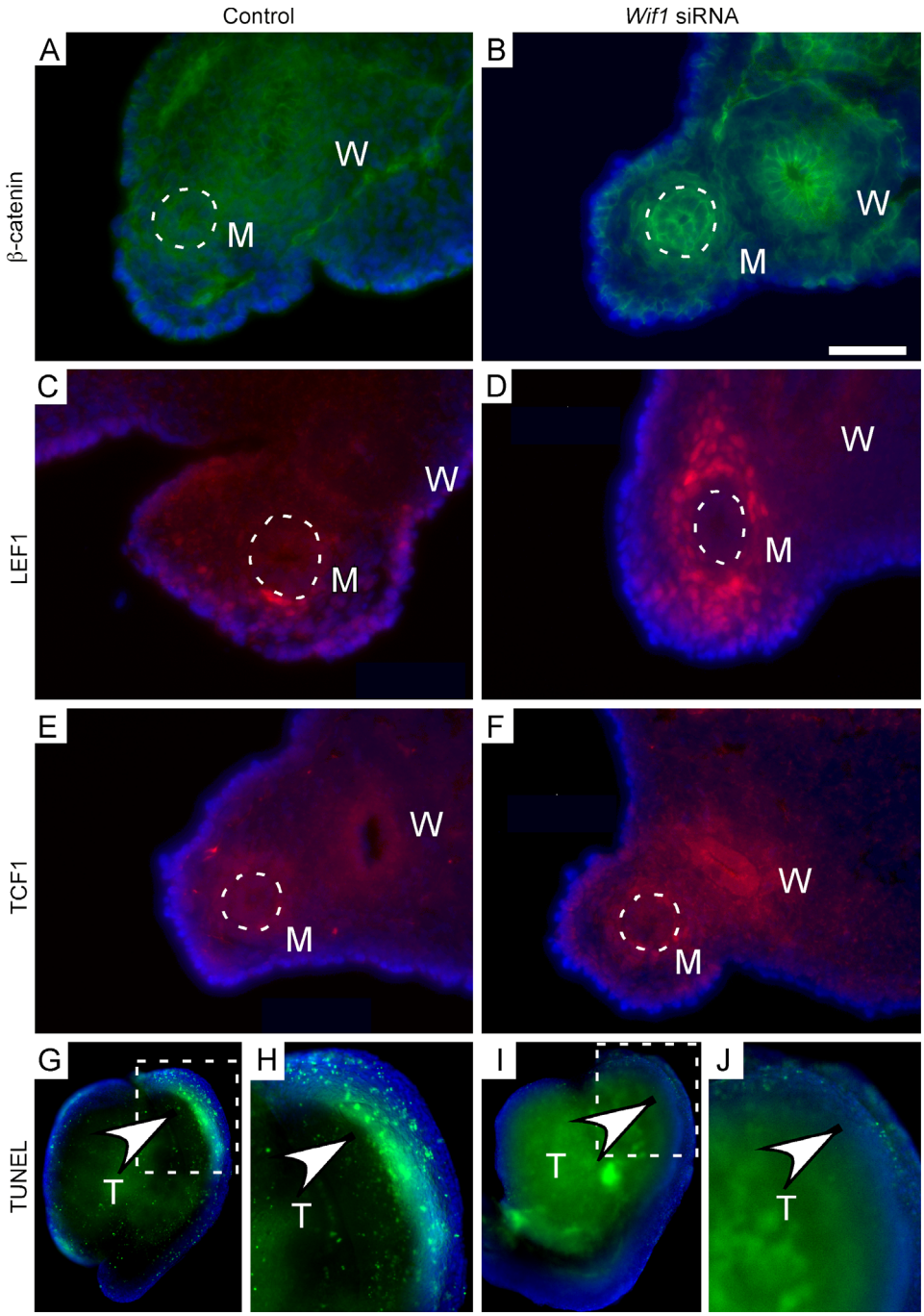

Fig. 4. Downstream activities of β-catenin are induced by *Wif1* knockdown. Expression of β-catenin ((A) and (B)) and its regulated genes, TCF1 ((C) and (D)) and LEF1 ((E) and (F)), were analyzed by immunofluorescence in frozen sections in either control siRNA-treated or *Wif1* siRNA-treated E13.5 male UGRs at 60 h post transfection. The degree of apoptosis in the MD was analyzed by determining the number of TUNEL-positive cells located mostly at the rostral tip (arrowhead) of the UGRs treated with either control siRNA (G) or with *Wif1* siRNA (I). White boxed areas in (G) and (I) are shown at higher magnification in (H) and (J). *M*=MD, *W*=Wolffian duct, *T*=testis. MD epithelium is outlined with a dashed line. Nuclei are stained with DAPI. Bar=100 μm.

caudal end, which regresses last (Picon, 1969; Tsuji et al., 1992). The whole mount in situ hybridizations clearly demonstrate regression of the MD as reflected by the absence or reduced level of staining for *Wnt7a* (Fig. 3C and D). In contrast, when the UGRs were exposed to a cocktail of *Wif1*-specific siRNAs, significant retention of the MDs was observed (Fig. 3B). In some UGRs, *Wnt7a* expression was observed along the whole length of the MD (Fig. 3E), but in others caudal expression was most prominent (Fig. 3F). After arbitrarily dividing the MD into three vertical segments, a quantitative comparison of the rate of MD retention between the control and *Wif1* siRNA-treated UGRs revealed a statistically significant difference (Fig. 3G) in each segment, confirming that knockdown of *Wif1* expression negatively impacts MD regression.

To rule out the possibility that MD retention might be due to an indirect effect of *Wif1* siRNA knockdown on the expression of either *Mis* or *Misr2*, the experiment was repeated and the URGs were examined by whole mount in situ hybridization with probes specific for *Mis* and *Misr2*. Neither *Mis* (Fig. 3H and J) nor *Misr2* (Fig. 3I and K) mRNA expression in the gonads appeared to be affected in the *Wif1* siRNA-treated UGRs compared to controls. *Misr2* expression in the MD mesenchyme, while limited to the caudal regions in the control-treated UGRs undergoing regression, was also observed in the retained MD regions of the *Wif1* siRNA-treated UGRs. In the face of MD regression in controls, changes in *Misr2* expression are more difficult to interpret in the *Wif1* knockdown UGRs because its expression is observed as an artifact of MD retention.

We next examined WNT/β-catenin signaling in control and *Wif1* knockdown male UGRs before MD regression is normally completed. Although membranous β-catenin was observed in the control UGRs, increased β-catenin signal was observed in both the mesenchymal cells surrounding the MD and the MD epithelial cells of *Wif1* siRNA-treated UGRs (Fig. 4A and B). Confirmation of increased nuclear β-catenin activity with *Wif1* siRNA was observed by strongly increased expression of LEF1 (Fig. 4C and D) and a more subtle increase in TCF expression (Fig. 4E and F). However, the induction of both these β-catenin-regulated genes appeared limited to the MD mesenchyme cells, suggesting that the increased β-catenin expression resulting from *Wif1* knockdown was not affecting nuclear β-catenin activity in the MD epithelial cells. These results would also suggest that during normal MD regression, MIS-induced WIF1 suppresses WNT/β-catenin signaling in the MD mesenchyme by an autocrine mechanism.

Because MIS-induced apoptosis in the Müllerian duct epithelium is a key phenotypical event in male MD regression, the impact of knocking down *Wif1* mRNA on apoptosis was investigated. TUNEL staining for UGRs after *Wif1* siRNA (Fig. 4G and H) versus negative control siRNA treatment (Fig. 4I and J) revealed a significantly increased distribution of apoptotic cells along the cranial portion of the ridges in the control siRNA treated UGRs (mean 211 ± 9 SEM vs. 32 ± 3 SEM; $p=0.0009$) at 60 h post transfection, confirming that induced WNT signaling negatively affects MD regression.

Discussion

The development of the male or female sex phenotype from the bipotential embryonic gonads has fascinated reproductive biologists for nearly a century. Aside from its inherent allure, it is also important to understand sex differentiation because genitourinary disorders are the most common in human development and of significant clinical importance to human health and quality of life. These include congenital uterine anomalies, cryptorchidism, intersex anomalies, hypospadias, and exstrophies, which are currently treated by surgical intervention, and in some cases combined with hormonal therapy, with varying degrees of success. Thus, the basic developmental mechanisms underlying gonadal and reproductive tract development have to be further investigated in order to understand more fully the underlying etiologies of these genitourinary anomalies with the goal of developing better and more rational methods to treat these patients. For example, the basic mechanisms and the complex coordination of regression, retention and selective differentiation of the Müllerian and Wolffian ducts are not completely understood. Our results reported here showing that inhibition of WNT signaling in male UGRs after MIS binds to its receptor, MISR2, in the MD mesenchyme provide an important contribution to the study of the key mechanisms governing the regression of MD epithelial cells.

We show that inducing WNT signaling by blocking MIS-mediated induction of *Wif1* with subsequent accumulation of β-catenin can result in partial MD retention and support our previous findings that male mice with constitutively activated β-catenin in the MD mesenchyme have a focal MD retention phenotype (Tanwar et al., 2010). Conversely, another report using the same *Misr2-Cre* allele indicated that β-catenin in the MD mesenchyme is required for regression (Kobayashi et al., 2011), supporting a previous study that suggested accumulation of β-catenin was required for MD regression (Allard et al., 2000). Taken together, these studies suggest an intricately regulated balance of WNT/β-catenin signaling may be crucial for normal regression of MD in males and that too much or too little β-catenin activity might in fact interfere with MD regression (Tanwar et al., 2010). A similar phenomenon has been observed during lung development, where altering the dosage of β-catenin activity in vivo by knockout or constitutively activated expression can have profound effects on epithelial cell differentiation (Mucenski et al., 2003, 2005; Okubo and Hogan, 2004). Another possibility is that inhibition of *Wif1* expression might be affecting WNT signaling pathways that do not involve canonical β-catenin activity, but rather those that involve the planar cell or Ca2+ pathways, or even the orphan receptor tyrosine kinase, ROR2, an important mechanism required for WNT5A-driven alveolarization in the lung (Loscertales et al., 2008).

A key finding from our studies shown in Fig. 4, suggests that decreased *Wif1* expression (i.e., more WNT signaling) appears to increase cellular β-catenin levels in both the MD epithelial cells and in the surrounding mesenchymal cells but induced nuclear expression of the proteins encoded by the WNT target genes, *Lef1* and *Tcf1,* was only observed in the mesenchymal cells. Although *Wif1* expression appears to be limited to the MD mesenchyme, it is a secreted factor that binds to WNT proteins and inhibits their binding to cell surface receptors, and subsequent intracellular paracrine WNT signaling in both the mesenchyme and adjacent epithelium. These results support the observation that LEF1 expression is observed in the mesenchyme of β-catenin intact UGRs but not in β-catenin knockout UGRs (Kobayashi et al., 2011). We speculate that the effects of WIF1 on canonical β-catenin activity appear to be autocrine. These results do not preclude a paracrine function for WIF1 in the epithelium but do suggest that if there is one, it probably does not require β-catenin-mediated transcriptional activation.

In addition to *Wif1*, our analyses comparing *Misr2* heterozygous with *Misr2* knockout UGRs showed that the expression of many other genes was changed by loss of MIS signaling (GSE38009). We chose *Wif1* for further analyses because of our interest in β-catenin signaling during Müllerian duct differentiation. *Msx2* and *Dlx5* are two other genes highly induced by MIS signaling in the UGRs that we expect to study further. The induction of *Msx2,* a homeobox gene that encodes the muscle segment homeobox homolog 2 protein, in the male MDs during regression has been previously shown in chicken embryos (Ha et al., 2008). Although loss of *Msx2* has pleiotropic

effects in mice, it does not appear to affect fertility (Satokata et al., 2000), which would be expected in males with retained MDs (Mishina et al., 1996). However, its loss does appear to exacerbate the deleterious effect of diethylstilbestrol on Müllerian duct development (Yin et al., 2006) and conditional postnatal knockout of both *Msx1* and *Msx2* leads to defective uterine implantation (Daikoku et al., 2011). *Dlx5*, another homeobox gene that encodes Distal-less homeobox 5 protein, has been implicated in craniofacial, limb, and bone development (Kraus and Lufkin, 2006), but there are no reports linking *Dlx5* to MD development or regression.

We examined whether evidence of MD retention could be observed in both neonatal and 4 week old male *Wif1*−/− mice (Kansara et al., 2009) but did not observe any MD-derived tissues in these mice (data not shown). The *Wif1*−/− mice have yet to provide an overt phenotype other than an increased susceptibility to develop osteosarcomas, which is very surprising given the number of diverse processes that WIF1 has been implicated in modulating, including lung development (Xu et al., 2011), hematopoiesis (Schaniel et al., 2011), and carcinogenesis (Ramachandran et al., 2012). A recent report showed that *Wif1* expression in the mesenchyme of the urogenital sinus is regulated by androgens and that it enhances prostatic development (Keil et al., 2012). However, they also reported no obvious prostate phenotype was observed in the *Wif1*−/− mice as well. The disparity between the results from the knockdown organ culture experiments shown in Fig. 3 and the absent *Wif*−/− knockout mouse phenotype is reminiscent of an earlier report showing that, although knockdown of matrix metalloproteinase 2 (MMP2) inhibited MD regression in organ culture, the *Mmp2*−/− male mice did not have retained MD tissues (Roberts et al., 2002). Those authors speculated that functional redundancy of the MMP family could be mitigating the loss of MMP2 on UGR development in vivo, which could also be an issue in the *Wif1*−/− mice with the several other WNT inhibitors available to replace some WIF1 function. Resolution of these discrepancies with the numerous critical activities attributed WIF1 and the lack of a *Wif1*−/− phenotype is eagerly awaited.

In summary, we have reported gene expression analyses of murine UGRs during MD regression with and without MIS signaling in order to understand the mechanisms driving a fundamental aspect of sexual differentiation. We chose *Wif1* for follow up studies and showed for the first time that MIS induces the expression of the *Wif1* gene, which encodes an inhibitor of WNT signaling. Confirmatory studies showed that knockdown of *Wif1* mRNA expression interferes with MD regression. Like the role of β-catenin itself during MD regression, we hypothesize that an exquisitely tuned amount of *Wif1* expression is required for normal reproductive tract development.

Acknowledgments

We would like to thank Dr. Igor Dawid and Martha Rebbert at NICHD for generously sharing their *Wif1*−/− mice with us. We are indebted to Dr. Richard Behringer at MD Anderson for providing us with the *Misr2-Cre* and *Misr2-LacZ* mice. These studies were supported by NIH grants to JMT (OD012206) and to PKD (CA017393) and by a DOD grant to PKD (W81XWH-12-1-0342).

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